AD_____

Award Number: W81XWH-07-1-0124

TITLE: Novel Therapies for Acinetobacter Osteomyelitis

PRINCIPAL INVESTIGATOR: Edward M. Schwarz, Ph.D.

CONTRACTING ORGANIZATION: University of Rochester
Rochester, NY 14642

REPORT DATE: January 2009

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-01-2009	Final	17 Jan 2007 – 31 Dec 2008

4. TITLE AND SUBTITLE

Novel Therapies for Acinetobacter Osteomyelitis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0124

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Edward M. Schwarz, Ph.D.

Clinton Murray, MD, MAJ, MC

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Rochester
Rochester, NY 14642

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
FORT DETRICK, MD 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Osteomyelitis (OM) from multidrug-resistant (MDR) *Acinetobacter* has emerged in >30% of combat-related injuries in Iraq and Afghanistan. While most of strains are sensitive to colistin, the drug is not availible in bone void fillers for local high-dose delivery. To address this we developed a mouse model with M strains isolated from wounded military personnel. In contrast to *S. aureus* OM, which is osteolytic and characterized by biofilm in necrotic bone, *A. baumani* results in blastic lesions that do not contain apparent biofilm. We also found that mice mount a specific IgG response against 3 proteins (40, 47 & 56KDa) re of the strain used, suggesting that these may be immunuo-dominant antigens. PCR for the *A. baumannii* specific *parC* gene confirmed a 100% infection rate 75% of the MDR strains, and *in vitro* testing confirmed that all strains were sensitive to colistin. We also developed a real-time quantitative PCR (RTQ-PCR that could detect as few as 10 copies of *parC* in a sample. To demonstrate the efficacy of colistin prophylaxis in this model, mice were treated with either pa colistin (0.2mg colistinmethate i.m. for 7 days), local colistin (PMMA bead impregnated with 1.0mg colistin sulfate), or an unloaded PMMA bead control. W parenteral colistin failed to demonstrate any significant effects vs. the placebo, the colistin PMMA bead significantly reduced the infection rate such that onl of the mice had detectable levels of *parC* at 19 days ($p<0.05$ vs. i.m. colistin and placebo).

15. SUBJECT TERMS
Multi-Drug Resistant, *Acinetobacter baumannii*, Osteomyelitis, Colistin

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	27	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

INTRODUCTION: It has been well established from current causulty data of our military operations in the Middle East that the ratio of serious injuries to fatal casualties far exceeds that of previous conflicts. Among these serious injuries, war wound infection and osteomyelitis (OM) appear to be of greatest concern. Most alarming is the incidence of multidrug-resistant (MDR) Acinetobacter species. This comes as a major surprise since this pathogen has been reported in less than 2% of nosocomial infections within the United States, but has emerged in over 30% of admitted deployed soldiers. An additional problem is that while there are some effective antibiotics against Acinetobacter (i.e. Colistin and Imipenem), they are not availible in bone void fillers that are primarily used to treat OM caused by Staphylococcus. To address this urgent need we propose a collaboration that will take advantage of the first quantitative animal model of implant-associated OM developed for Staphylococcus, and clinical isolates of MDR Acinetobacter obtained from our soldiers. To achive these goals we will test the **hypotheses** that: 1) prophylatic chemotherapy with Acinetobacter-specific antibiotics can prevent the establishment of Acinetobacter OM; 2) incorporation of Acinetobacter-specific antibiotics into polymethylmethacrylate bone void filler prevents OM in a contaminated wound; and 3) specific antibodies are raised against common immuno-dominant antigens during the establishment of Acinetobacter OM. Our experimental approach to test these hypotheses in embodied in the following Specific Aims.

BODY: The following is a summary of the research accomplishments associated with the tasks outlined in the approved Statement of Work. Specific details of all the experimental methods and data analyses are provided in the appended manuscript, which has been accepted for publication in the Journal of Orthopaedic Research.

Specific Aim 1. To develop quantitative outcome measures to assess Acinetobacter osteomyelitis *in vivo*. We have developed the first small animal model of Acinetobacter OM that can be quantified by: i) RTQ-PCR of a bacterial gene (*parC*) standardized to a host gene (β*-actin*); ii) micro-CT analyses of osteolysis; and iii) histology using five MDR Acinetobacter strains from soldiers who were treated for OM following battle field injuries.

Specific Aim 2. To evalute the efficacy of Acinetobacter-specific antibiotics (Colistin) as prophylatic chemotherapies and local therapy in bone void filler. Using the aforementioned A. baumannii strains and quantitative mouse model, we evaluated mice treated with either parenteral colistin (0.2mg colistinmethate i.m. for 7 days), local colistin (PMMA bead impregnated with 1.0mg colistin sulfate), or an unloaded PMMA bead control. While the parenteral colistin failed to demonstrate any significant effects vs. the placebo, the colistin PMMA bead significantly reduced the infection rate such that only 29.2% of the mice had detectable levels of *parC* at 19 days ($p<0.05$ vs. i.m. colistin and placebo).

Specific Aim 3. To identify the immuno-dominant antigens of Acinetobacter. Another important strategy to prevent these infections is the development of an Acinetobacter vaccine. An effective vaccine would contain parts of the Acinetobacter that stimulates the production of antibodies against critical parts of the bacteria know as immuno-dominant antigens. Using the aforementioned A. baumannii strains and quantitative mouse model, we found that mice mount a specific IgG response against 3 proteins (40, 47 & 56KDa) regardless of the strain used, suggesting that these may be immunuo-dominant antigens.

KEY RESEARCH ACCOMPLISHMENTS:

-We have established the first quantitative small animal model of Acinetobacter osteomyelitis, which can be used to: study microbial pathogenesis, evaluated novel antibiotics, and develop novel vaccines to treat our soldiers.

-We have generated “proof-of-principle” data that colistin impregnated PMMA bead prevent the establishment of A. baumannii osteomyelitis, which warrants further evaluation in a clinical trial.

-We have identified three immuno-dominant antigens that are expressed by all strains of A. baumannii tested. These antigens could be used to develop a protective vaccine.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

1) Manuscripts (see below). 2) A commercial source of colistin impregnated PMMA beads.

CONCLUSION: We propose that colistin impregnated PMMA beads are the solution to *A. baumannii* osteomyelitis, and should be used to treat wounded soldiers at the time of surgery.

REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in *Science, Military Medicine*, etc.).

Daniel P. Crane, Kirill Gromov, Clinton K. Murray, Regis, J. O’Keefe, and Edward M. Schwarz, Colistin-Impregnated Bone Cement Beads Prevent Multi-Drug Resistant Acinetobacter Osteomyelitis. ***J. Bone Mineral Res.*** 2008; 23:S432.

Crane DP, Gromov K, Li D, Søballe K, Wahnes C, Büchner H, Hilton MJ, O’Keefe RJ, Murray CK, Schwarz EM. Efficacy of Colistin Impregnated Beads to Prevent Multi-drug Resistant A. baumannii Implant-Associated Osteomyelitis. ***J Orthop Res*** *2009;In Press.*

APPENDICES: See attached preprint of Crane DP, et al. ***J Orthop Res*** *2009; In Press.*

SUPPORTING DATA: See attached preprint of Crane DP, et al. ***J Orthop Res*** *2009; In Press.*

Journal of Orthopaedic Research

Efficacy of Colistin Impregnated Beads to Prevent Multi-drug Resistant A. baumannii Implant-Associated Osteomyelitis

Journal:	*Journal of Orthopedic Research*
Manuscript ID:	JOR-08-0434.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Crane, Daniel; University of Rochester, Orthopaedics Gromov, Kirill; University of Rochester, Orthopaedics Li, Dan; University of Rochester, Orthopaedics soballe, kjeld; University Hospital of Aarhus, Orthopedics; University Hospital of Aarhus, Orthopedics Wahnes, Christian; Heraeus Medical GmbH Buechner, Hubert; Heraeus Medical GmbH Hilton, Matthew; University of Rochester, Orthopaedics O'Keefe, Regis; University of Rochester, Orthopaedics Murray, Clinton; Brooke Army Medical Center, Fort Sam Houston, Infectious Disease Service, Dept. of Medicine Schwarz, Edward; University of Rochester, Dept. of Orthopaedics
Keywords:	Osteomyelitis, multidrug-resistant , Acinetobacter , colistin , military casulties

Efficacy of Colistin Impregnated Beads to Prevent Multi-drug Resistant *A. baumannii* Implant-Associated Osteomyelitis

*Daniel P. Crane[1], *Kirill Gromov[1,2], Dan Li[1], Kjeld Søballe[2],

Christian Wahnes[3], Hubert Büchner[3], Matthew J. Hilton[1], Regis J. O'Keefe[1],

Clinton K. Murray[4,5], and Edward M. Schwarz[1,6]

[1]The Center for Musculoskeletal Research, University of Rochester,
Rochester, NY
[2]The Department of Orthopedics, Aarhus University Hospital, Aarhus, Denmark
[3]Research & Development, Heraeus Medical GmbH, Wehrheim (Germany)
[4]Infectious Disease Service, Dept. of Medicine, Brooke Army Medical Center, Fort Sam Houston, San Antonio, TX
[5]Disclaimer: The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the Department of the Army, Department of Defense or the US government. This work was prepared as part of their official duties and, as such, there is no copyright to be transferred.

[6]To whom correspondence should be addressed:
Dr. Edward M. Schwarz
The Center for Musculoskeletal Research
University of Rochester Medical Center
601 Elmwood Avenue, Box 665, Rochester, NY 14642
Phone 585-275-3063, FAX 585-756-4727
E-mail: Edward_Schwarz@URMC.Rochester.edu.

*These authors contributed equally to this work.

Key Words: Multi-Drug Resistant, *Acinetobacter baumannii*, Osteomyelitis, Colistin

Summary:

Osteomyelitis (OM) from multidrug-resistant (MDR) *Acinetobacter* has emerged in >30% of combat-related injuries in Iraq and Afghanistan. While most of these strains are sensitive to colistin, the drug is not availible in bone void fillers for local high-dose delivery. To address this we developed a mouse model with MDR strains isolated from wounded military personnel. In contrast to *S. aureus* OM, which is osteolytic and characterized by biofilm in necrotic bone, *A. baumannii* OM results in blastic lesions that do not contain apparent biofilm. We also found that mice mount a specific IgG response against 3 proteins (40, 47 & 56KDa) regardless of the strain used, suggesting that these may be immunuo-dominant antigens. PCR for the *A. baumannii* specific *parC* gene confirmed a 100% infection rate with 75% of the MDR strains, and *in vitro* testing confirmed that all strains were sensitive to colistin. We also developed a real-time quantitative PCR (RTQ-PCR) assay that could detect as few as 10 copies of *parC* in a sample. To demonstrate the efficacy of colistin prophylaxis in this model, mice were treated with either parenteral colistin (0.2mg colistinmethate i.m. for 7 days), local colistin (PMMA bead impregnated with 1.0mg colistin sulfate), or an unloaded PMMA bead control. While the parenteral colistin failed to demonstrate any significant effects vs. the placebo, the colistin PMMA bead significantly reduced the infection rate such that only 29.2% of the mice had detectable levels of *parC* at 19 days ($p<0.05$ vs. i.m. colistin and placebo).

Introduction

It has been well established from current combat-related injuries during US military operations in Iraq and Afghanistan that the ratio of serious injuries to fatal casualties exceeds that of previous conflicts.[1] Orthopaedic trauma comprises the vast majority of these war wounds, as 70% of casualties involve the

musculoskeletal system, 26% are fractures, and 82% of the fractures are open. [2-5] Thus, it is not surprising that the incidence of osteomyelitis (OM) in combat-related extremity injuries is between 2% to 15%, and is of great concern.[6-8] Most alarming is the incidence of infections caused by multidrug-resistant (MDR) *Acinetobacter* species, which can be difficult to cure in some settings.[9-11] Surprisingly this pathogen has been reported in less than 2% of nosocomial infections within the United States, but has emerged in over 30% of admitted deployed soldiers.[10]

In contrast to *Staphylococcus*, which is responsible for >80% of OM infections,[12] *Acinetobacter baumannii-calcoaceticus* complex (ABC) are Gram-negative, non-fermentative, non-spore forming, strictly aerobic, oxidase-negative coccobacillary organisms. Additionally, infections caused by *Acinetobacter* appear to be hospital-acquired and not from an initial colonization of the injury. [13] Thus, one critical question involving *Acinetobacter* is whether or not they can produce osteolytic OM on their own, or if they are only present in super-infections with other microorganisms. Another important question is whether or not the MDR *Acinetobacter* OM can be effectively prevented with parenteral or local antibiotic therapy at the time of initial surgery. In support of this a clinical study has demonstrated that most MDR Acinetobacter strains are sensitive to colistin,[10] and that colistin heteroresistance primarily occurs in patients treated with colistin.[14] Thus, we aimed to test the hypotheses that: i) pure clinical isolates of MDR *Acinetobacter* can induce implant-associated OM, and ii) prophylactic colistin prevents these orthopaedic infections. To this end, we utilized a quantitative murine model of implant-associated OM, orginally developed for *S. aureus*,[15] in which an insect pin is innoculated with cultured bacteria and transcortically implanted through the tibia metaphysis. Given the well-established use of antibiotic impregnated bone cement to deliver high doses of drug locally,[16-18] we have developed colistin sulfate impregnated polymethylmethacrylate (PMMA) beads, since high dose parenteral administration of colistin is

limited by nephrotoxicity and neurotoxicity.[19] With this system, here we provide the first evidence that *A. baumannii* can induce OM in the absence of other pathogens, however these lesions are blastic rather than osteolytic and do not contain apparent biofilms in necrotic bone. Moreover, local but not parenteral pharmacological doses of colistin are capable of preventing the establishment of MDR Acinetobacter implant-associated OM.

Methods

Bacterial strains Four clinical isolates of *Acinetobacter baumannii* with confirmed resistance to amikacin, ampicillin, aztraeonam, ceftriaxone, ciprofloxacin, gentamicin, imipenem, tobramycin and vancomycin were obtained from wounded soldiers treated at the Brooke Army Medical Center at Fort Sam Houston, San Antonio, under Institutional Review Board approved protocols. Bacterial strains were grown overnight at 250rpm and 37°C in Tryptic Soy broth (Sigma-Aldrich, St. Louis, MO). Strain sensitivity to colistin was determined by streaking overnight cultures on Trypic Soy agar plates containing 10μg/ml of colistinmethate (Paddock Laboratories, Inc, Minneapolis, MN).

DNA extraction, PCR cloning of parC and RTQ-PCR of Acinetobacter DNA DNA was extracted from all four strains using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). DNA primers (forward 5'-AAAAATCAGCGCGTACAGTG-3' and reverse 5'-CGAGAGTTTGGCTTCGGTAT-3') specific for the *Acinetobacter* topoisomerase gene *parC*, were used for PCR and RTQ-PCR as previously described.[20] To confirm the purity of the clinical isolates, 5 individual colonies from each strain were isolated and the *parC* gene was amplified and sequenced using an i-cycle PCR machine (Bio-Rad, Hercules, CA). The amplification protocol was as follows: 95°C pre-melt for 15min followed by 40 cycles of 95°C for 30s, 54°C for 30s and 72°C

for 30s. To generate a standard curve for RTQ-PCR, the 196bp *parC* fragment was cloned into the pTOPO2.1 cloning vector (Invitrogen, Carlsbad, CA), and 10-fold serial dilutions were used to perform Syber Green (Thermo Scientific) RTQ-PCR (Corbett Research, Sydney, AU). A similar curve was generated using the mouse *β-actin* primers (forward 5'-AGATGTGAATCAGCAAGCAG-3' and reverse 5'-GCGCAAGTTAGGTTTTGTCA-3'), to control for sample integrity as we have previously described.[15] In order to calculate the *parC* gene copies in a tibia sample, we first generated a standard curve with *A. baumannii* genomic DNA purified directly from an overnight culture. The standard curve was generated with 10-fold dilutions of the TOPO plasmid with *parC* insert. The mean of the 3 Ct values from each tibia sample were then plotted against this curve to extrapolate the number of *parC* genes (n= 24 mice/treatment group). This number was then normalized to *β-actin* and the data are presented as normalized *parC* gene copies per sample.

Colistin in vitro release kinetics PMMA beads (Heraeus Medical GmbH, Wehrheim, Germany) were impregnated with 1.0mg or 2.0mg colistin sulphate (Alpharma, Copenhagen, Denmark). To assess the release kinetics of the colistin following rehydration, the beads were introduced into capped vials and totally covered with 10mL of MilliQ water. Extraction was carried out at 37°C, and the water was exchanged every 24h. The concentration of released colistin sulphate in the extracts was determined by conductometric measurements with a WTW platinated platin electrode LTA 1 at 25°C. Temperature fluctuations were compensated using a WTW TFK 530 temperature electrode in parallel to the conductive electrode. A calibration curve with 40 points (R^2=0.999) at concentrations between 0 and 20g/L was recorded to calculate concentrations. All of the measured concentrations were within this range. Placebo beads were used as control, and the conductive values of the placebo beads were subtracted from detected signals before calculating colistin concentrations.

Surgery and antibiotic treatments All animal studies were performed under University of Rochester Committee for Animal Resources approved protocols. The implant-induced OM surgeries were performed on 6-8 week-old C57Bl/6 female mice as previously described.[15] Briefly, mice were anesthetized with ketamine (100mg/kg) and xylazine (10mg/kg), shaved, and the skin was cleansed with 70% ethanol. A small incision was made in the skin on the medial side of the left leg to expose the tibial metaphysis. OM was induced via transcoritcal insertion of a 0.25mm insect pin (Fine Science Tools, Foster City, CA) that was dipped into an overnight culture of *A. baumannii* or *S. aureus* (Xen29), which contaminated the pin with ~$2.5x10^5$ colony forming units (CFU) as determined by votexing the inoculated pins in PBS and plating out the contents on agar. For prophylactic treatment, mice (n = 24) received either: 1) a control PMMA bead lacking antibiotic, 2) a PMMA bead impregnated with 1.0mg colistin sulphate, or 3) intramuscular (i.m.) injection of 0.2mg (~10mg/kg) of colistinmethate every day for 7 days as previously described in a mouse pneumonia model.[21] The 5mm PMMA beads were implanted adjacent to the pin at the time of surgery and were secured with a suture through the skin and muscle. The parenteral colistinmethate was given at the time of surgery and for the next 7 days with no bead. All mice were sacrificed for analysis on day 19.

Radiology. Longitudinal plain film radiographs were obtained using a Faxitron Cabinet x-ray system (Faxitron, Wheeling, IL, USA) as we have previously described.[22] Micro-computed tomography (μCT) was performed on tibia after sacrifice at high-resolution (10.5 μm) (VivaCT 40; Scanco Medical AG, Basserdorf, Switzerland) to render 3D images as we have previously described.[23]

Histologic evaluation of OM. After μCT, the tibial samples were processed for decalcified histology and stained with hematoxylin and eosin (H&E), or Gram-stained as we have described previously.[24]

Serology. The generation of specific antibodies against *A. baumannii* proteins during the establishment of chronic OM was determined by western blotting as previously described.[15] Briefly, total protein extract was

obtained from a 100 ml culture of *A. baumannii*, strains BAMC 2, BAMC 3 and BAMC 4, using the Complete Bacterial Proteome Extraction Kit (Calbiochem, San Diego, CA). 20 µg of total *A. baumannii* protein per well was boiled in Laemelli loading buffer and separated in NuPAGE™ 10% Bis-Tris SDS Gels (Invitrogen, Carlsbad, CA) by electrophoresis, and transferred to a PVDF membrane (Millipore, Billerica, MA). The membrane was then cut into single lanes and blocked with PBS, 0.1% Tween 20 (PBST) and 5% non-fat dry milk for 1 hr at room temperature. Afterwards, each lane was incubated with a unique serum (10 µl serum in 5 ml of blocking buffer (PBST + 5% non-fat dry milk)) as the primary antibody, washed 3 times in 0.1% PBST, and then the strips were pooled and incubated with 1.5 µl HRP-conjugated goat anti-mouse IgG antibody (BioRad, Hercules, CA). The strips were then washed 3 times in PBST, 15 minutes each at room temperature. Finally, the strips were reassembled with the molecular weight marker strip and imaged with ECL+ (Amersham) chemiluminescence autoradiograph.

Results

A murine model of implant-associated MDR *A. baumannii* osteomyelitis. Having recently established a quantitative transtibial model of OM with *S. aureus*,[15] we aimed to utilize this same approach to develop the first mouse model of *A. baumannii* OM. Thus, we obtained four strains of *A. baumannii* that were isolated from soldiers wounded in the Middle East and screened them for antibiotic resistance. While these isolates displayed variable resistance to the most commonly used antibiotics in orthopaedic bone cement (gentamicin, tobramycin and vancomycin), they were all sensitive to colistin (Figure 1A). In order to assess the virulence of these strains in our mouse model, stainless steel insect pins were contaminated with an overnight culture of each strain and then surgically implanted into the tibiae of mice. The presence of infection was determined through amplification of the *A. baumannii* specific *parC* gene. While all of the mice survived these infections, Figure 1B

demonstrates the presence of chronic OM in 100% of the mice challenged with strains BAMC2, BAMC3 and BAMC4. Strain BAMC1 failed to establish infection in all of the mice tested and was therefore excluded from further experiments. Further evidence of this infection was demonstrated by the development of *A. baumannii* specific antibodies in the sera of challenged mice, which appeared around day 11 and recognized 3 protein antigens 40, 47 and 56KDa that were conserved in all four strains (Figure 1C).

Implant-associated MDR *A. baumannii* osteomyelitis is osteoblastic. One of the salient features of OM is osteolysis around the implant and the presence of biofilm in the adjacent necrotic bone and soft tissue. Thus, we examined the effects of *A. baumannii* OM in our model compared to *S. aureus* and identified several remarkable differences between these bacterial pathogens. Most striking was that in contrast to the osteolytic response to *S. aureus*, *A. baumannii* OM induces a robust osteoblastic bone formation response around the infected pin (Figures 2A-D). This dramatic difference in the host bone response to the bacteria was also evident in histology sections of the infected area, which confirmed the large osteolytic lesions in *S. aureus* OM filled with inflammatory tissue (Figure 2E), contrasted by the new woven bone adjacent to the pin tract (Figure 2F). Examination of Gram stained sections demonstrated another interesting difference between these pathogens in that *S. aureus* OM is always associated with the presence of biofilm in necrotic bone fragments,[25] such as that observed in Figure 2G, while we were unable to identify any biofilm in the necrotic tissue adjacent to the *A. baumannii* infected pins (Figure 2H). Although this negative finding is not conclusive, it raises the possibility that *A. baumannii* may persist as an intracellular pathogen in chronic OM.

Local prophylactic colistin prevents MDR A. baumannii OM. In order to evaluate the efficacy of prophylactic colistin in our model, we first developed a real time quantitative (RTQ) PCR assay to assess the *in vivo* bacterial load. Figure 3 demonstrates the sensitivity and specificity of this assay, which was able to

reproducibly detect as few as 10 *parC* copies in infected bone. Since we were interested in assessing the difference between parenteral and local colistin, we chose to utilize antibiotic impregnated PMMA beads as the carrier. In pilot studies we found that the 5mm beads could be readily implanted adjacent to the infected pin (Figure 4A), while 1cm beads were too big to be used in this mouse model (Figure 4B). We then evaluated the *in vitro* release kinetics of the colistin from the 5mm beads to ensure an appropriate biodistribution of the drug over time (Figure 4C). These studies demonstrated that 40-50% of the loaded colistin-sulphate is steadily released into solution from the beads over the first 5 days, which is when the drug would be most effective in killing bacteria initiating the chronic infection. As there were no marked differences between the 1mg and 2mg doses in these studies, we moved forward to *in vivo* challenge experiments with 1.0mg colistin-sulphate impregnated PMMA beads. BAMC-1 was excluded from this study due to its low virulence as shown in Figure 1B. Following sacrifice on day 19, the bacterial load (Figure 5A), incidence of infection for all strains (Figure 5B), and the incidence of infection for each strain (Figure 5C), was determined by RTQ-PCR. Infection rates were determined to be 75% (18/24), 71% (17/24 and 33% (8/24) for mice receiving a placebo bead, intramuscular colistinmethate injection or colistin-sulphate impregnated PMMA bead, respectively. While the results failed to demonstrate any significant effects of parenteral colistin versus placebo control, local colistin significantly reduced the incidence of chronic OM versus both placebo and parenteral colistin. Moreover, when we analyzed the strains individually, it was clear that most of the infections in the colistin bead group were caused by BAMC-2, suggesting that this strain may have heteroresistance or may be more virulent than the other strains.

Discussion

Infections caused by MDR pathogens have long been recognized to be a very serious problem in medicine, requiring vigilance when prescribing antibiotic therapy. Most recently this subject has received

tremendous attention, as epidemiology studies seem to indicate that prosthetic infections may be on the rise, [26 27] and methicillin-resistant *Staphylococcus aureus* (MRSA) has surpassed HIV as the most deadly pathogen in the United States.[28] Therefore, the emergence of MDR *Acinetobacter* OM in orthopaedic trauma patients was initially proposed to be a situation requiring urgent attention.[9-11] However, more recent clinical experiences in dealing with this problem suggest that MDR *Acinetobacter* OM can be managed effectively, but may be the predecessor to more serious super-infections including MRSA.[7,29] Of these orthopedic injuries in veterans of Operation Iraqi Freedom and Operation Enduring Freedom (OIF/OEF) we found that Gram-negative pathogens predominate early, and are replaced with staphylococci after treatment, despite nearly universal use of Gram-positive therapy. More specifically, *Acinetobacter* species were present in 70% of OM cases at presentation, but only 5% of reoccurring OM. In contrast, *S. aureus* was only present in 13% of initial OM, while 53% of reoccurrences were infected with Staphylococcus, and 31% of the cases were MRSA. Most notable were the type III diaphyseal tibial fractures, of which 13 out of the 35 patients studied had union times of >9 months that appeared to be associated with infection, and 4 that ultimately required limb amputation due to infectious complications. These new findings underscore the importance of effective early treatment of MDR *Acinetobacter* OM, and warrant investigation of the cause and effect relationship between initial Gram negative infections that evolve into catastrophic MRSA OM. To address these issues, here we investigated the nature of MDR *A. baumannii* OM versus that of *S. aureus*, and evaluated the efficacy of local high dose colistin to prevent infection from a contaminated tibial implant in a mouse model.

Although we found MDR *Acinetobacter* to be highly infectious as expected (Figure 1), we were surprised by several features of *A. baumannii* OM that are remarkably distinct from *S. aureus* infection of bone (Figure 2). The first is that *A. baumannii* induces blastic lesions, in contrast to the osteolytic lesions most

commonly associated with OM. Although we have no information on the mechanism by which the bacteria stimulates bone formation, this may occur via induction of anabolic factors (i.e. BMPs, Wnts), and/or the down-regulation of antagonists (i.e. noggin, sost, dkk), in a similar manner as that observed in osteoblastic tumors.[30] The other major difference that we found was the absence of biofilm and any histological evidence of colonized necrotic bone in mice infected with *A. baumannii*, which is always present in chronic *S. aureus* OM. One possible explanation of these results, which needs to be explored in a focused investigation, is that *A. baumannii* persists as an obligate intracellular pathogen. In so doing, the bacteria would be immune privileged from humoral immunity (Figure 1C), which should clear extracellular bacteria from the infection site. Additionally, the absence of extracellular bacteria and their pathogen-associated molecular patterns (PAMPs),[31] would lead to decreased Toll-like receptor (TLR) activation of innate immunity and osteoclast activation, which causes osteolysis.[32-35]

Another interesting speculation that is brought by these data and the recent clinical studies on OM cases from OIF/OEF is the possibility that *S. aureus* infections are opportunistic, and in some cases may depend on an initial colonization by Gram negative bacteria such as *Acinetobacter*. This scenario further stresses the importance of early eradication of peri-implant infection following orthopaedic surgery, which is most effectively achieved with local drug therapy via antibiotic impregnated bone cement,[16-18] and potentially antibiotic coated implants [36,37]. Given that most MDR *Acinetobacter* strains are sentive to colistin,[10] and that PMMA beads can be readily impregnated with colistin and steadily release the drug over time (Figure 4), we evaluted this mode of local prophylaxis versus standard parenteral colistinmethane (Figure 5). As predicted, the local treatment, which presumably leads to higher drug concentration levels at the site of infection, was significantly better in preventing MDR *Acinetobacter* OM. Considering the vast independent clinical experience with antibiotic impregnated

PMMA beads and colistin, and the absence of an accepted large animal model of OM that simulates the soft tissue injury associated with war wounds, we find that these results support the evaluation of colistin impregnated immprengnated PMMA beads, or absorbable materials that do not require removal, in a clinical trial to evaluate their efficacy in clearing MDR *Acinetobacter* OM and reoccuring infections.

Acknowledgments

The authors would like to thank Laura Yanoso for technical assistance with the micro-CT and Krista Scorsone for technical assistance with the histology. This work was supported by research grants from the US Army Medical Research Acquisition Activity (USAMRAA), Orthopaedic Trauma Research Program (OTRP) W81XWH-07-1-0124, and the National Institutes of Health PHS awards AR48681, DE17096, AR52674, AR51469, AR46545, AR54041 and AR53459.

References

1. Covey DC. Iraq war injuries. *Orthopedics 2006;29-10:884-6.*

2. Owens BD, Kragh JF, Jr., Macaitis J, Svoboda SJ, Wenke JC. Characterization of extremity wounds in Operation Iraqi Freedom and Operation Enduring Freedom. *J Orthop Trauma 2007;21-4:254-7.*

3. Owens BD, Kragh JF, Jr., Wenke JC, Macaitis J, Wade CE, Holcomb JB. Combat wounds in operation Iraqi Freedom and operation Enduring Freedom. *J Trauma 2008;64-2:295-9.*

4. Covey DC. Combat orthopaedics: a view from the trenches. *J Am Acad Orthop Surg 2006;14-10 Suppl:S10-7.*

5. Covey DC, Aaron RK, Born CT, Calhoun JH, Einhorn TA, Hayda RA, Levin LS, Mazurek MT, Murray CK, Powell ET, Schwarz EM, Wenke JC. Orthopaedic war injuries: from combat casualty care to definitive treatment: a current review of clinical advances, basic science, and research opportunities. *Instr Course Lect 2008;57:65-86.*

6. Murray CK, Hsu JR, Solomkin JS, Keeling JJ, Andersen RC, Ficke JR, Calhoun JH. Prevention and management of infections associated with combat-related extremity injuries. *J Trauma 2008;64-3 Suppl:S239-51.*

7. Yun HC, Branstetter JG, Murray CK. Osteomyelitis in military personnel wounded in Iraq and Afghanistan. *J Trauma 2008;64-2 Suppl:S163-8; discussion S8.*

8. Acinetobacter baumannii infections among patients at military medical facilities treating injured U.S. service members, 2002-2004. *MMWR Morb Mortal Wkly Rep 2004;53-45:1063-6.*

9. Murray CK, Hospenthal DR. Treatment of multidrug resistant Acinetobacter. *Curr Opin Infect Dis 2005;18-6:502-6.*

10. Davis KA, Moran KA, McAllister CK, Gray PJ. Multidrug-resistant Acinetobacter extremity infections in soldiers. *Emerg Infect Dis 2005;11-8:1218-24.*

11. Abbo A, Navon-Venezia S, Hammer-Muntz O, Krichali T, Siegman-Igra Y, Carmeli Y. Multidrug-resistant Acinetobacter baumannii. *Emerg Infect Dis 2005;11-1:22-9.*

12. Darouiche RO. Treatment of infections associated with surgical implants. *N Engl J Med 2004;350-14:1422-9.*

13. Scott P, Deye G, Srinivasan A, Murray C, Moran K, Hulten E, Fishbain J, Craft D, Riddell S, Lindler L, Mancuso J, Milstrey E, Bautista CT, Patel J, Ewell A, Hamilton T, Gaddy C, Tenney M, Christopher G, Petersen K, Endy T, Petruccelli B. An outbreak of multidrug-resistant Acinetobacter baumannii-calcoaceticus complex infection in the US military health care system associated with military operations in Iraq. *Clin Infect Dis 2007;44-12:1577-84.*

14. Hawley JS, Murray CK, Jorgensen JH. Colistin heteroresistance in acinetobacter and its association with previous colistin therapy. *Antimicrob Agents Chemother 2008;52-1:351-2.*

15. Li D, Gromov K, Soballe K, Puzas JE, O'Keefe RJ, Awad H, Drissi H, Schwarz EM. Quantitative mouse model of implant-associated osteomyelitis and the kinetics of microbial growth, osteolysis, and humoral immunity. *J Orthop Res 2008;26-1:96-105.*

16. Marks KE, Nelson CL, Jr., Schwartz J. Antibiotic-impregnated acrylic bone cement. *Surg Forum 1974;25-0:493-4.*

17. Wininger DA, Fass RJ. Antibiotic-impregnated cement and beads for orthopedic infections. *Antimicrob Agents Chemother 1996;40-12:2675-9.*

18. Parvizi J, Saleh KJ, Ragland PS, Pour AE, Mont MA. Efficacy of antibiotic-impregnated cement in total hip replacement. *Acta Orthop 2008;79-3:335-41.*

19. Falagas ME, Kasiakou SK. Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. *Clin Infect Dis 2005;40-9:1333-41.*

20. Hamouda A, Amyes SG. Development of highly ciprofloxacin-resistant laboratory mutants of Acinetobacter baumannii lacking topoisomerase IV gene mutations. *J Antimicrob Chemother 2006;57-1:155-6.*

21. Montero A, Ariza J, Corbella X, Domenech A, Cabellos C, Ayats J, Tubau F, Borraz C, Gudiol F. Antibiotic combinations for serious infections caused by carbapenem-resistant Acinetobacter baumannii in a mouse pneumonia model. *J Antimicrob Chemother 2004;54-6:1085-91.*

22. Zhang X, Schwarz EM, Young DA, Puzas JE, Rosier RN, O'Keefe RJ. Cyclooxygenase-2 regulates mesenchymal cell differentiation into the osteoblast lineage and is critically involved in bone repair. *J Clin Invest 2002;109-11:1405-15.*

23. Koefoed M, Ito H, Gromov K, Reynolds DG, Awad HA, Rubery PT, Ulrich-Vinther M, Soballe K, Guldberg RE, Lin AS, O'Keefe R J, Zhang X, Schwarz EM. Biological Effects of rAAV-caAlk2 Coating on Structural Allograft healing. *Mol Ther 2005;12-2:212-8.*

24. Ito H, Koefoed M, Tiyapatanaputi P, Gromov K, Goater JJ, Carmouche J, Zhang X, Rubery PT, Rabinowitz J, Samulski RJ, Nakamura T, Soballe K, O'Keefe R J, Boyce BF, Schwarz EM. Remodeling of cortical bone allografts mediated by adherent rAAV-RANKL and VEGF gene therapy. *Nat Med 2005;11-3:291-7.*

25. Brady RA, Leid JG, Calhoun JH, Costerton JW, Shirtliff ME. Osteomyelitis and the role of biofilms in chronic infection. *FEMS Immunol Med Microbiol 2008;52-1:13-22.*

26. Kurtz SM, Lau E, Schmier J, Ong KL, Zhao K, Parvizi J. Infection Burden for Hip and Knee Arthroplasty in the United States. *J Arthroplasty 2008.*

27. Pulido L, Ghanem E, Joshi A, Purtill JJ, Parvizi J. Periprosthetic joint infection: the incidence, timing, and predisposing factors. *Clin Orthop Relat Res 2008;466-7:1710-5.*

28. Klevens RM, Morrison MA, Nadle J, Petit S, Gershman K, Ray S, Harrison LH, Lynfield R, Dumyati G, Townes JM, Craig AS, Zell ER, Fosheim GE, McDougal LK, Carey RB, Fridkin SK. Invasive methicillin-resistant Staphylococcus aureus infections in the United States. *Jama 2007;298-15:1763-71.*

29. Johnson EN, Burns TC, Hayda RA, Hospenthal DR, Murray CK. Infectious complications of open type III tibial fractures among combat casualties. *Clin Infect Dis 2007;45-4:409-15.*

30. Virk MS, Lieberman JR. Tumor metastasis to bone. *Arthritis Res Ther 2007;9 Suppl 1:S5.*

31. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol 2001;2-8:675-80.*

32. Bi Y, Collier TO, Goldberg VM, Anderson JM, Greenfield EM. Adherent endotoxin mediates biological responses of titanium particles without stimulating their phagocytosis. *J Orthop Res 2002;20-4:696-703.*

33. Bi Y, Seabold JM, Kaar SG, Ragab AA, Goldberg VM, Anderson JM, Greenfield EM. Adherent endotoxin on orthopedic wear particles stimulates cytokine production and osteoclast differentiation. *J Bone Miner Res 2001;16-11:2082-91.*

34. Bi Y, Van De Motter RR, Ragab AA, Goldberg VM, Anderson JM, Greenfield EM. Titanium particles stimulate bone resorption by inducing differentiation of murine osteoclasts. *J Bone Joint Surg Am 2001;83-A-4:501-8.*

35. Nalepka JL, Lee MJ, Kraay MJ, Marcus RE, Goldberg VM, Chen X, Greenfield EM. Lipopolysaccharide found in aseptic loosening of patients with inflammatory arthritis. *Clin Orthop Relat Res 2006;451:229-35.*

36. Antoci V, Jr., King SB, Jose B, Parvizi J, Zeiger AR, Wickstrom E, Freeman TA, Composto RJ, Ducheyne P, Shapiro IM, Hickok NJ, Adams CS. Vancomycin covalently bonded to titanium alloy prevents bacterial colonization. *J Orthop Res 2007.*

37. Parvizi J, Antoci V, Jr., Hickok NJ, Shapiro IM. Selfprotective smart orthopedic implants. *Expert Rev Med Devices 2007;4-1:55-64.*

Figure Legends

Figure 1. Characterization of MDR *A. baumannii* strains in the murine model of implant-associated osteomyelitis. (A) Four MDR *A. baumannii* clinical isolates were screened for colistin sensitivity by plating on agar medium with (top) and without (bottom) 10μg/ml of colistinmethate. The absence of bacterial growth confirms that all strains were colistin sensitive. (B) The ability of the clinical isolates to establish chronic OM was evaluated in the murine model as described in Materials and Methods. Mice (n=8) were infected with contaminated pins and their tibiae were harvested on day 19 for *par C* PCR, and the 196bp product was resolved in a 2% agarose gel stained with ethidum bromide. Lanes 1-8 are from strain BAMC1, lanes 9-15 are from strain BAMC2, lanes 16-23 are from strain BAMC3, lanes 24-31 are from strain BAMC4, lane 32 is the pTOPO-*parC* positive control and lane 33 is the no template negative control. (C) Mice (n=4) were bled on the indicated day following infection, and their sera were used as the primary antibody in western blots of total cell extract of the *A. baumannii* strains. A representative autoradiograph is shown demonstrating the presence of nonspecific IgG antibodies that were present in all of the sera (arrowhead) and whose titer did not increase over time, and specific IgG antibodies against *A. baumannii* proteins present in all the strains that initially appeared on day 11 and whose titer increased thereafter in all of the mice tested (arrows).

Figure 2. Differential host bone responses to *S. aureus* vs. *A. baumannii* infection. Representative radiology (A-D) and histology (E-H) of *S. aureus* (A, B, E & G) and *A. baumannii* (C, D, F & H) OM in the mouse model are shown. Of note is the remarkable osteolysis that is induced by *S. aureus* as evidenced in the radiolucent x-ray (A), lytic lesion in the 3D micro-CT image (B) and the inflammatory tissue surrounding the pin tract in H&E stained histology at 20x magnification (E). The necrotic bone fragments in these lesions (boxed region in E) were laden with biofilm that could be readily identified in parallel Gram stained sections at 40x magnification (arrow in G). In contrast, *A. baumannii* OM was characterized by blastic lesions that were radiodense on x-ray (C), and contained copious amounts of new bone around the infected pin (D & F). This made it difficult to identify the pin tract in histology sections, which had to be confirmed by injecting pathology marking ink (Newcomer Supply Inc., Middleton, WI) into the pin hole before processing (arrow in F). Furthermore, Gram staining failed to identify any foreign material in the necrotic bone fragments adjacent to the pin tract (H).

Figure 3. Sensitivity and Specificity of RTQ-PCR to quantify bacterial load in *A. baumannii* OM. A real time quantitative PCR assay to detect the *A. baumannii* bacterial load in the infected tibiae was developed by generating standard curves from 10-fold dilutions of pTOPO-*parC*, which was standardized to mouse DNA (pTOPO-*βactin*) as we have previously described for S. aureus.[15] Syber green RTQ-PCR was performed with *parC* (A-C) or β-actin (D-F) specific primers. The primary threshold cycle values (CT) data of the dilutions (A & D) were used to generate a standard curve for each template (blue dots in B & E). Then the DNA from the infected tibiae described in Figure 1 was extracted and amplified with the same *parC* and *β-actin* specific primers, and extrapolated to the standard curve (red dots in B & E), allowing for an estimate of bacterial load that is presented as *parC* copies/*β-actin* copies per sample. Given that contamination can be a problem with real time PCR at >35 cycles, and that we could reproducible quantify 10 copies of parC at 30.1 cycles, we set this value as

the upper threshold limit to detect our PCR products. The purity was confirmed using a melt curve, which identified the predicted single peak for the *parC* (C) and *β-actin* (F) PCR products respectively.

Figure 4. Colistin impregnated PMMA Beads. X-rays were taken of mice following implantation of a 5mm (A) and 1cm (B) PMMA bead adjacent to a transcortical pin. The *in vitro* colistin release kinetics of 5mm PMMA beads impregnated with 1.0 or 2.0mg of colistin-sulfate was determined over the course of 5 days (C). The data are presented as the mean +/- SD (n=5) of the cumulative colistin recovered in solution at the indicated time, such that the values for each day are added to the previous values to give a cumulative total from time 0. No significant differences between the 1.0 and 2.0mg dose were detected by t-test with Bonferroni correction.

Figure 5. Local but not parenteral colistin prevents *A. baumannii* implant-associated osteomyelitis. Immediately prior to transtibial implantation of a pin contaminated with the indicated strain of *A. baumannii*, mice ((n=8) with strains BAMC-2, 3 and 4; 24 total mice per group) received either: i) a sterile PMMA bead (placebo), ii) daily 200μg intramuscular injection of colistinmethate for seven days, or iii) a PMMA bead impregnated with 1mg of colistin-sulphate. Following sacrifice on day 19, *par C* real-time quantitative PCR was used to measure bacterial load (A), and infection rates (B & C). Of the mice receiving the placebo, 18 out of 24 were infected, in mice receiving intramuscular colistinmethate injection, 17 out of 24 mice were infected and in mice receiving a PMMA bead impregnated with 1mg of colistin-sulphate, 8 out of 24 mice were infected. While no significant effects of IM colistin treatment were observed verses the placebo control, the colistin impregnated bead significantly reduced the incidence of chronic OM compared to placebo (#) and IM colistin (*; $p > 0.05$ using Fisher's exact test) (B). However, when the strains were analyzed individually (C) it was clear that BAMC2 was responsible for most of the chronic infections in the mice treated with local colistin.

Figure 1

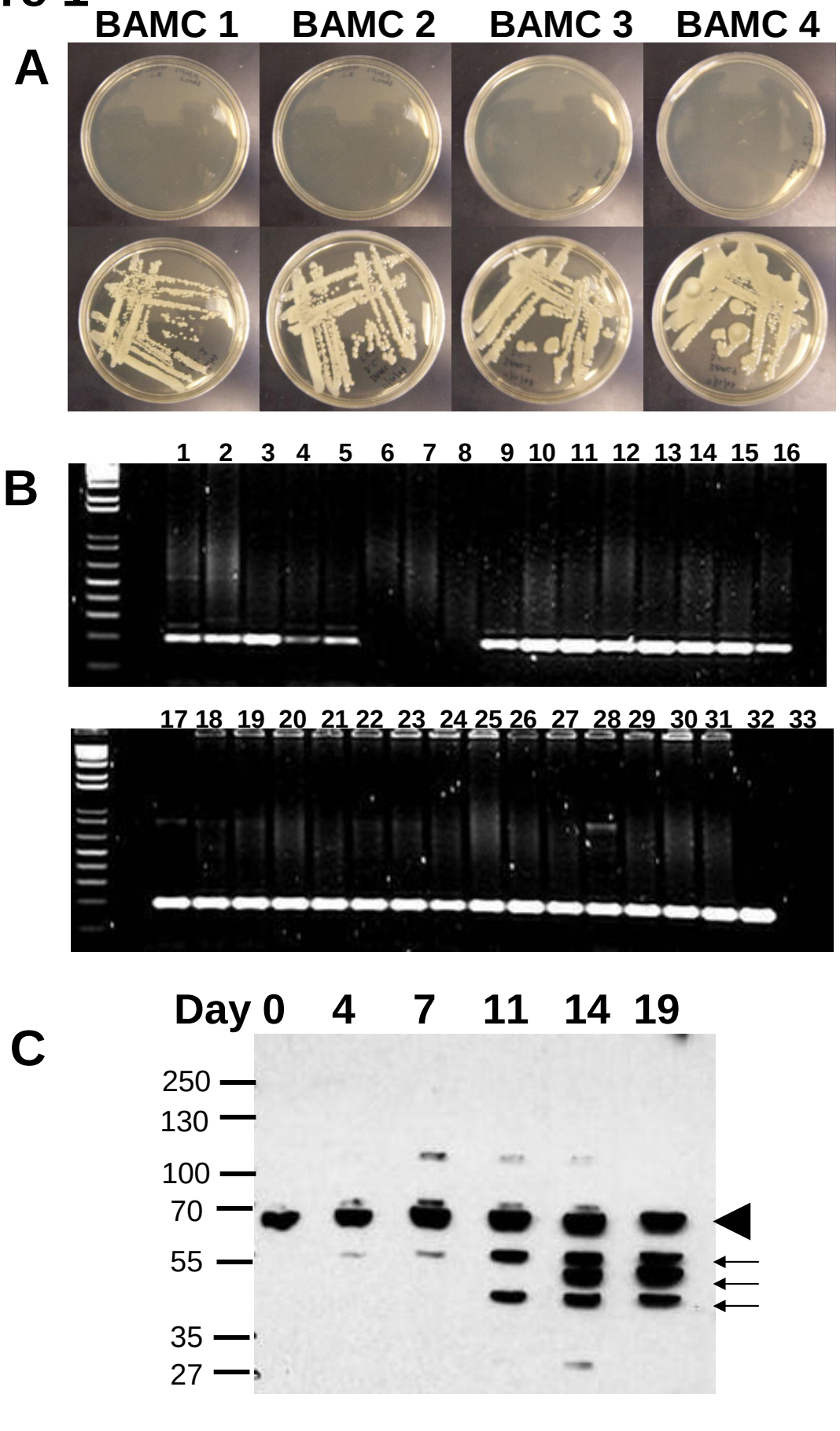

A
BAMC 1
BAMC 2
BAMC 3
BAMC 4
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33
C
Day 0 4 7 11 14 19
250
130
100
70
55
35
27

Figure 2

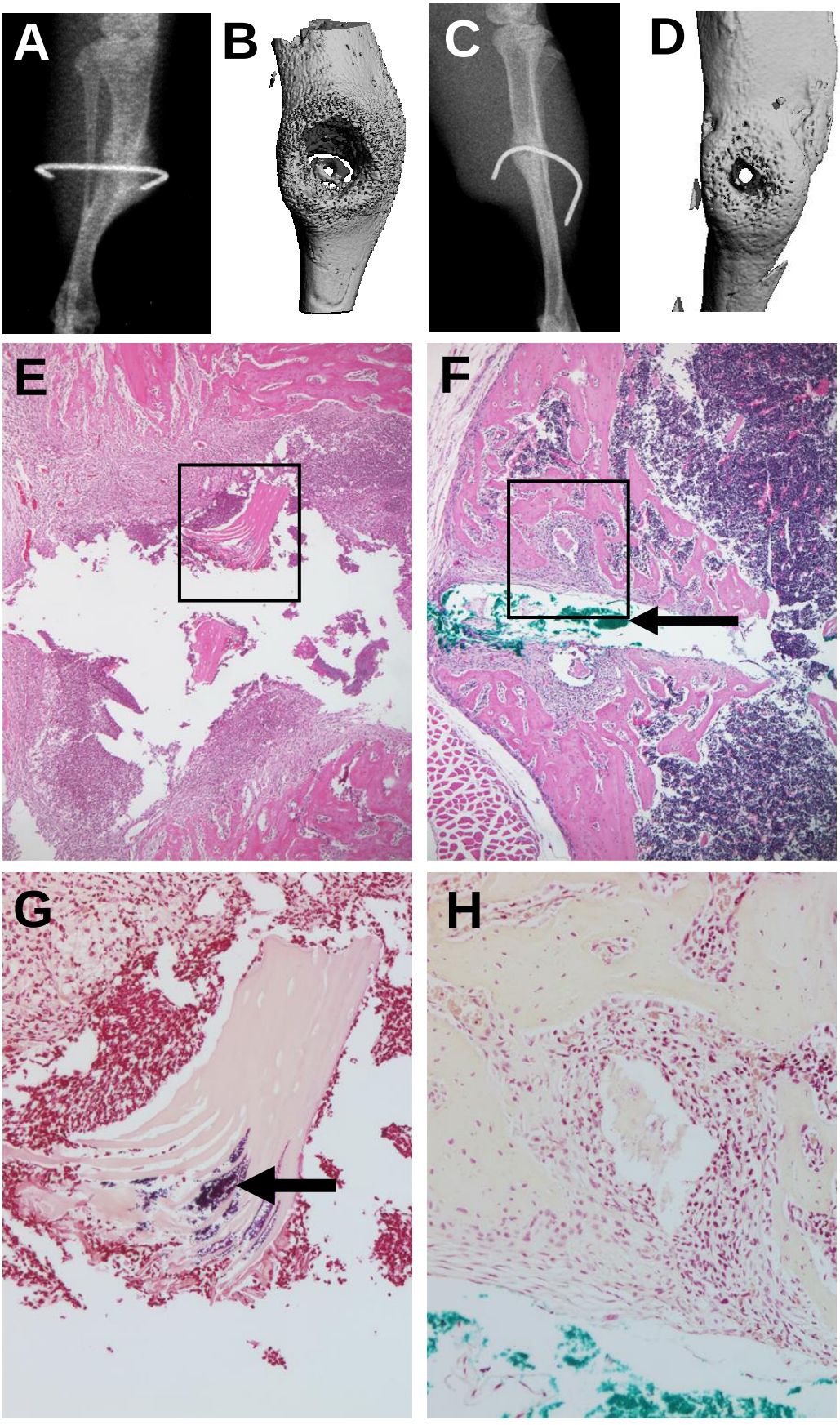


Figure 3

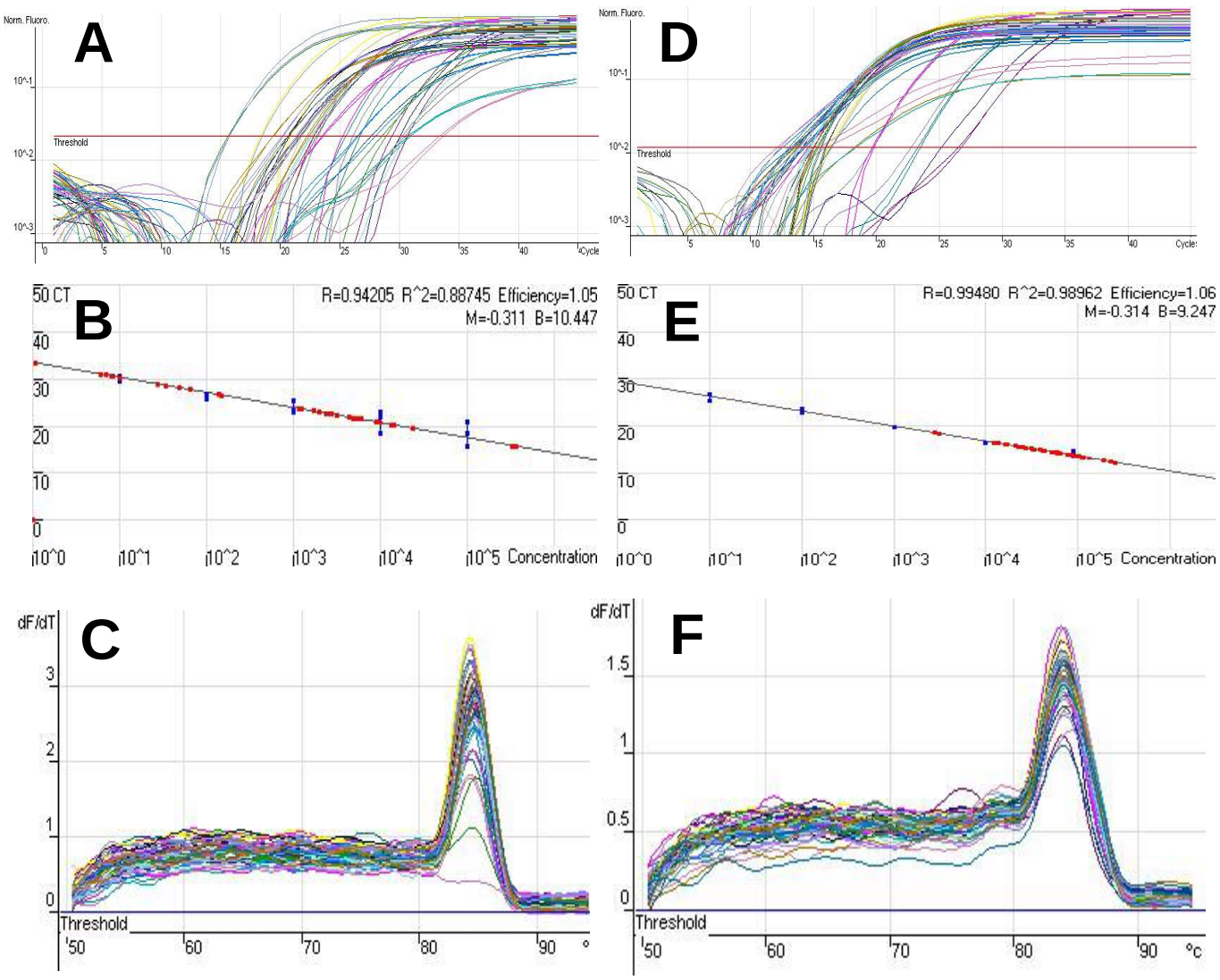
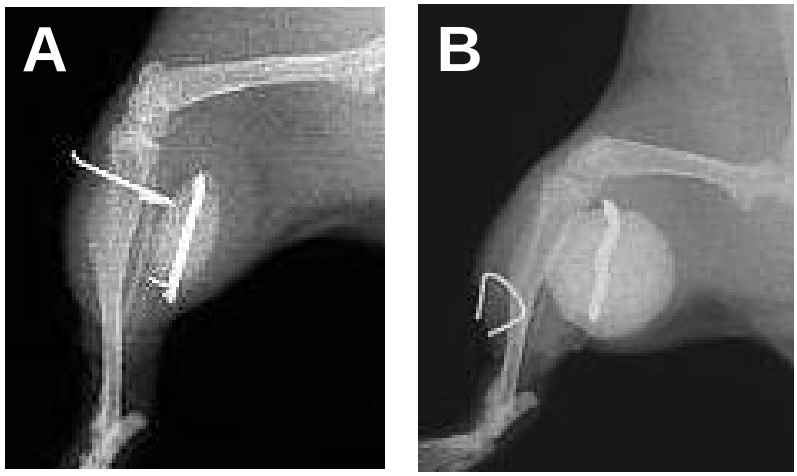

A
Norm. Fluoro.
Threshold
Cycle
D
Norm. Fluoro.
Threshold
Cycle
B
50 CT
R=0.94205 R^2=0.88745 Efficiency=1.05
M=-0.311 B=10.447
Concentration
E
50 CT
R=0.99480 R^2=0.98962 Efficiency=1.06
M=-0.314 B=9.247
Concentration
C
dF/dT
Threshold
°
F
dF/dT
Threshold
°C

Figure 4


A
B

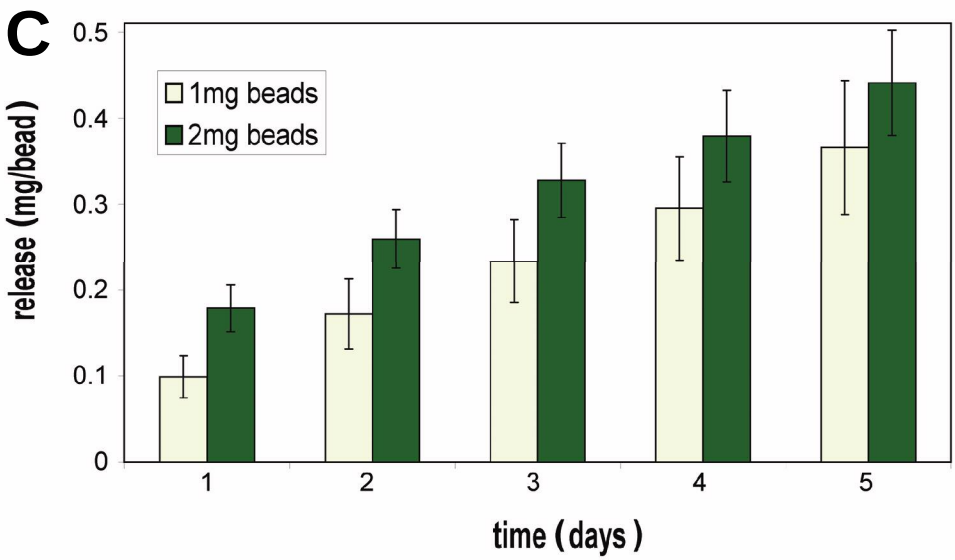
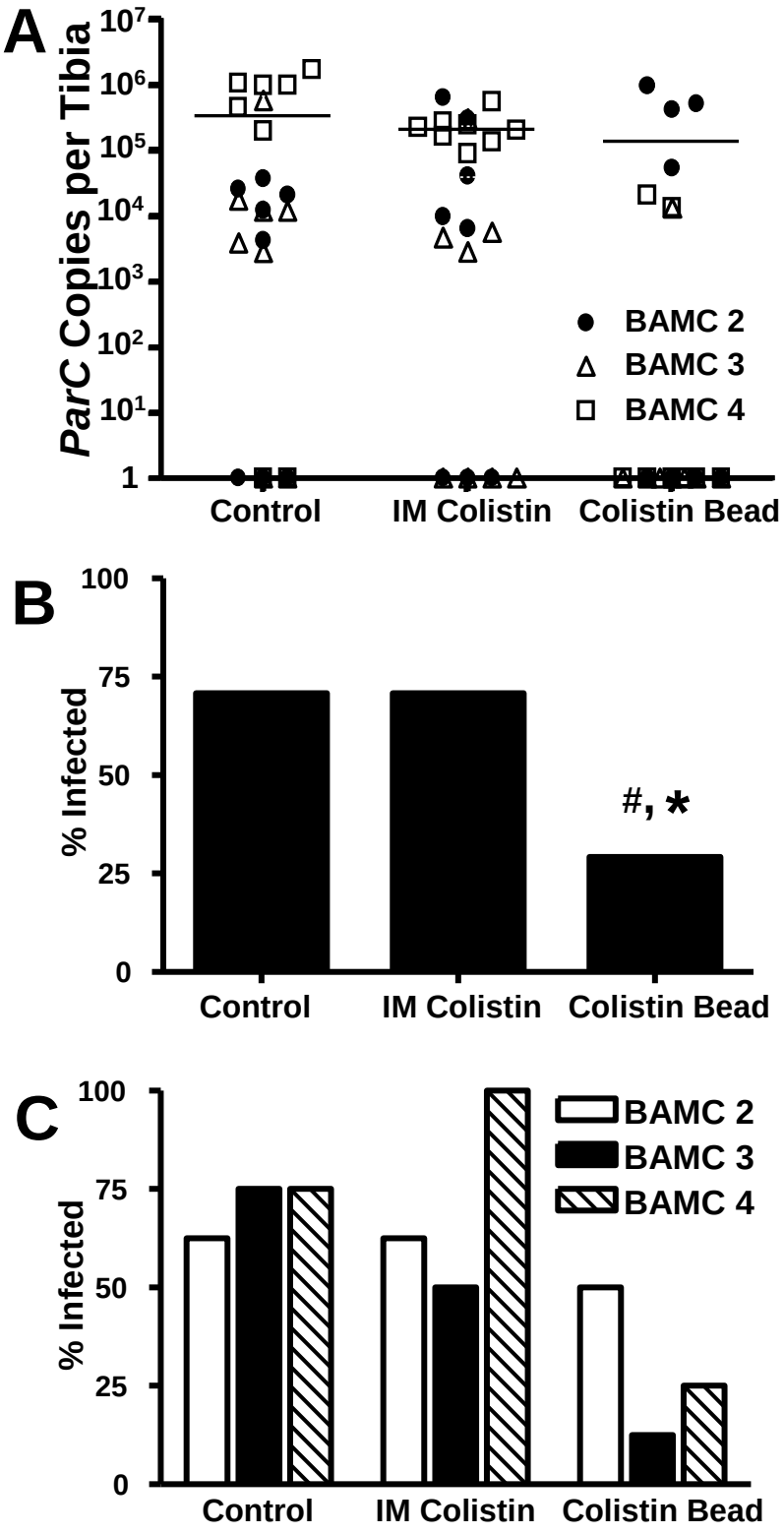

C
0.5
0.4
0.3
0.2
0.1
0
release (mg/bead)
1mg beads
2mg beads
1
2
3
4
5
time (days)

Figure 5


A
ParC Copies per Tibia
10^7
10^6
10^5
10^4
10^3
10^2
10^1
1
BAMC 2
BAMC 3
BAMC 4
Control
IM Colistin
Colistin Bead
B
% Infected
100
75
50
25
0
#, *
Control
IM Colistin
Colistin Bead
C
% Infected
100
75
50
25
0
BAMC 2
BAMC 3
BAMC 4
Control
IM Colistin
Colistin Bead